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The safety and efficacy of five prototype, live anthrax vaccines were studied in Hartley guinea pigs and CBA/J and A/J mice. Two of the strains, Bacillus anthracis FD111 and FD112, are Aro⁻ mutants derived by transposon mutagenesis UM23-1. B. subtilis strains PA1 and PA2 contain a recombinant plasmid, pPA101 or pPA102 respectively, that carries the gene from B. anthracis encoding protective antigen (PA). The final strain, B. subtilis PA7, was isolated in these studies from B. subtilis DB-104 transformed with pPA101. All five strains were less virulent in guinea pigs and A/J and CBA/J mice than the toxinogenic

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nonencapsulated B. anthracis veterinary vaccine Sterne strain. A/J and CBA/J inbred mice represent mouse strains that are innately susceptible and resistant, respectively, to the Sterne strain. These differences in susceptibility are due to differences in ability to produce complement component 5. In guinea pigs immunization with PA1 or PA2 vegetative cells or PA7 spores protected 95% or greater from an intramuscular spore challenge of the virulent, "vaccine-resistant" B. anthracis Ames strain. Strain PA2 vegetative cells and strain PA7 spores were as effective as the Sterne strain in Sterne-resistant CBA/J mice, protecting 70% of the mice from Ames spore challenge. Immunization with FD111 or FD112 vegetative cells fully protected guinea pigs from challenge. Immunization with FD111 cells protected up to 100% of CBA/J mice and up to 70% of A/J mice.

Immunization Against Anthrax with Aromatic-dependent (Aro$^-$) Mutants of *Bacillus anthracis* and with Recombinant Strains of *Bacillus subtilis* Producing Anthrax Protective Antigen

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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ABSTRACT

The safety and efficacy of five prototype, live anthrax vaccines were studied in Hartley guinea pigs and CBA/J and A/J mice. Two of the strains, Bacillus anthracis FD111 and FD112, are Aro$^-$ mutants derived by Tn916 mutagenesis of B. anthracis UM23-1. B. subtilis strains PA1 and PA2 contain a recombinant plasmid, pPA101 or pPA102 respectively, that carries the gene from B. anthracis encoding synthesis of protective antigen (PA). The final strain, B. subtilis PA7, was isolated in these studies from B. subtilis DB104 transformed with pPA101. All five strains were less virulent in guinea pigs and A/J and CBA/J mice than the toxinogenic, nonencapsulated B. anthracis veterinary vaccine Sterne strain. A/J and CBA/J inbred mice represent mouse strains that are innately susceptible and resistant, respectively, to the Sterne strain. These differences in susceptibility are due to differences in ability to produce complement component 5. In guinea pigs immunization with PA1 or PA2 vegetative cells or PA7 spores protected $\geq$95% from an intramuscular spore challenge of the virulent, "vaccine-resistant" B. anthracis Ames strain. Strain PA2 vegetative cells and strain PA7 spores were as effective as the Sterne strain in Sterne-resistant CBA/J mice, protecting 70% of the mice from Ames spore challenge. Immunization with FD111 or FD112 vegetative cells fully protected guinea pigs from

challenge. Immunization with FD111 cells protected up to 100% of CBA/J mice and up to 70% of A/J mice.

Two anthrax vaccines are licensed for use in the United States. The veterinary vaccine is a suspension of viable spores of the toxigenic, nonencapsulated Bacillus anthracis Sterne strain (22). Its use occasionally results in necrosis at the inoculation site and, rarely, death. The human vaccine consists of aluminum hydroxide-adsorbed supernatant material from fermentor cultures of another toxigenic, non-encapsulated B. anthracis strain, V770-NP1-R (19). Undesirable characteristics of this vaccine include the need for numerous boosters and the reduced ability to protect laboratory animals against certain virulent strains of B. anthracis (15,23), such as the Ames strain.

We are evaluating various living and chemical candidate vaccines to develop improved, safe, and efficacious prophylaxis against anthrax (10). Recently, we cloned the B. anthracis protective antigen (PA) gene into the asporogenic B. subtilis strain 1S53, by using the vector pUB110 (9). Two of the clones, PA1 and PA2, carried recombinant plasmids pPA101 and pPA102, respectively, and produced PA in broth cultures at levels equal to or greater than those of the parent B. anthracis Sterne strain. In preliminary experiments, immunization with PA1 protected guinea pigs and mice from lethality by a virulent spore challenge with B. anthracis strain Vollum 1B and partially protected rats from lethality by an anthrax toxin challenge (9,25). Although this preliminary evidence suggested that vegetative cells of PA-producing B. subtilis recombinants immunized against anthrax, there were no data on the efficacy of spores of PA-producing B. subtilis. In

the present studies, we transformed a spore-forming B. subtilis strain, DB104, with the plasmid from strain PA1. Spores from one of the clones isolated, PA7, were tested along with PA1 and PA2 vegetative cells as live vaccines in mice and guinea pigs against parenteral challenge by spores of B. anthracis strain Ames.

The mutagenesis of B. anthracis with Tn916 was also previously described (11). Two of the insertion mutants were deficient in the synthesis of aromatic compounds (18), and they were designated FD111 and FD112. We tested B. anthracis FD111 and FD112 in these studies as prototype live vaccines in guinea pigs and mice against B. anthracis Ames spore challenge. The Ames strain was used for challenge in these studies since it more readily overcomes vaccine-stimulated resistance to lethal infection with B. anthracis than do strains such as Vollum 1B (15, 23).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1.

Isolation and characterization of sporogenic *B. subtilis* PA-producing clones. Competent cells of the sporogenic *B. subtilis* strain DB104 were transformed with plasmid pPA101 DNA isolated from *B. subtilis* PA1 (4,5,9), as described previously (9). After isolating kanamycin-resistant (Km^r) colonies and identifying of clones producing PA on immunoassay agar (17), we isolated and examined plasmid DNA from randomly selected recombinants (3,9,16). One of the clones, PA7, was selected and further characterized. The serological and biological activities of the PA produced by *B. subtilis* PA7 were assayed by the methods described for *B. subtilis* PA1 and PA2 (2,6,9,13,20,24). PA production was assayed in cultures grown in defined *B. anthracis* R medium (20), with 0.8% yeast extract (RYE), or in RYE with 50 mM HEPES (RYEH, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid).

Preparation of vegetative cells for immunization. Vegetative *B. subtilis* cells were prepared for immunization by a modification of a previously described method (9). For the *B. subtilis* isogenic strains PA1, PA2, and BST1, approximately 10^3 CFU were inoculated into a 250-ml Erlenmyer flask containing 100 ml of brain heart infusion (BHI, Difco Laboratories, Detroit, Mich.) with 10 μg of kanamycin per ml, and the culture was incubated overnight with rapid shaking at 37°C. Twenty ml of culture was then inoculated

into a 2-liter Erlenmyer flask containing 1000 ml of BHI with kanamycin, and the culture was shaken for 4 h at 37°C. The cells were pelleted by centrifugation at 10,000 x g, washed once with phosphate-buffered saline (PBS), suspended in PBS with 12% glycerol, and frozen at -70°C. Survival during frozen storage was monitored by periodic assay of viability on plates of sheep blood agar to ensure correct dosages of viable cells, or spores, during immunization. B. anthracis Sterne and ΔSterne-1 vegetative cells were prepared similarly, except they were grown in BHI without kanamycin.

B. anthracis FD111 and FD112 cells were also prepared for immunization studies. Plates of sheep blood agar consisting of 5% sheep blood in blood agar base (Difco) with tetracycline (10 μg per ml) were inoculated with FD111 and FD112 and incubated overnight at 37°C. Isolated colonies were then inoculated into 250-ml flasks containing 50 ml of Aro growth medium (AGM) consisting of: brain heart infusion, 37 g; yeast extract, 10 g; L-tyrosine, 144 mg; L-phenylalanine, 85 mg; L-tryptophan, 265 mg; p-aminobenzoic acid, 20 mg; 2,3-dihydroxybenzoic acid, 10 mg; p-hydroxybenzoic acid, 10 mg; and deionized water, 1 liter. Tetracycline HCl was then added to a concentration of 10 μg per ml. The cultures were incubated for 12 h at 37°C with shaking at 100 rpm. One ml of each culture was inoculated into a 250-ml Nephelo sidearm culture flask (Bellco Glass, Inc., Vineland N.J.) containing 50 ml of AGM with tetracycline; 10 ml of each culture was inoculated into a 2-liter flask containing 500 ml AGM with tetracycline. All cultures

were incubated at 37°C with shaking until the absorbance at 540 nm (as measured on a Bausch and Lomb [Rochester, NY] Spectronic 21 spectrophotometer) of the cultures in the Nephlo flasks had increased 30-fold. The bacteria in the 500-ml cultures were then collected by centrifugation at 9,000 X *g* for 15 min. The cell pellets were resuspended in PBS, pelleted again, resuspended to 1% of the original culture volume in PBS with 12% glycerol, and dispensed (2 ml) into 12 X 75-mm polypropylene tubes and frozen at -70°C. B. anthracis UM23-1 cells for immunization were prepared similarly from cultures not containing tetracycline.

Preparation of spores for immunization. For preparation of B. subtilis PA7 spores, approximately 10^3 vegetative CFU were inoculated into 100 ml of Leighton Doi broth (14) with 10 *μg* of kanamycin per ml; the culture was incubated for 18 h with vigorous shaking at 37°C. Two-liter Erlenmyer flasks, each containing 200 ml of Leighton Doi broth, were inoculated with 5-ml amounts of the culture. After the cultures were shaken for 24 h at 37°C, 800 ml of sterile distilled water was added to each flask, and incubation with shaking was continued for 40 h. The cells and spores were pelleted at 10,000 x *g*, then resuspended in PBS containing 0.1% gelatin (PBSG). The cell suspension (10 ml) was added to 35-ml centrifuge tubes containing 8 ml of 60% Renografin (E. R. Squibb & Sons, Inc., Princeton, N.J.) overlayed with 14 ml of 50 % Renografin in PBSG. These discontinuous gradients were centrifuged in a Sorvall HB-4 hanging bucket rotor (Dupont, Wilmington, Del.) for 3 h at 12,000 x *g*. The spores, which formed a band at the interface

of the 50% and 60% Renografin layers, were removed by pipette and diluted with an equal volume of PBSG. The spores were pelleted at 10,000 x *g*, resuspended in PBSG containing 12% glycerol, and frozen at -70°C. *B. anthracis* spores from the Sterne and Ames strains and *B. subtilis* DB104 spores were prepared as above from Leighton-Doi medium not containing kanamycin.

Determination of generation time for *B. anthracis*. Nephelo sidearm culture flasks containing 25 ml of AGM (without tetracycline) were inoculated, in triplicate, with 10^2 to 10^3 CFU of *B. anthracis* UM23-1, FD111, or FD112. The cultures were incubated with shaking at 37°C. After cultures reached an Absorbance at 540 nm (A_{540}) of 0.01 (approximately 2 X 10^8 CFU/ml), turbidity measurements were performed as above at 0.5-h intervals. Generation times were the time required for A_{540} to double during logarithmic growth.

Reversion of *B. anthracis* FD111 and FD112 to the parental phenotype. One-liter Erlenmyer flasks containing 100 ml of AGM or AGM with tetracycline (10 μg/ml) were inoculated with approximately 10^2 CFU of *B. anthracis* FD111 or FD112. The cultures were incubated with shaking at 37°C for 48 h. Samples of the cultures were periodically tested for viability and growth characteristics on the following media with or without tetracycline: brain heart infusion agar (Difco), unsupplemented Brewer's agar (BA)(1,11), and Brewer's agar supplemented with phenylalanine, tyrosine, tryptophan, p-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and p-hydroxybenzoic acid added at the same concentrations as in AGM.

Experimental animals. Female Hartley guinea pigs (8) weighing

350 to 375 g at the beginning of the vaccination regimens, and CBA/J and A/J mice (25,26) weighing 25 g were used in the immunization studies. Female Hartley guinea pigs were also used in determinations of anthrax edema-producing toxin activity (21). Male Fischer 344 rats weighing 200 to 300 g were used to determine anthrax lethal toxin activity (6).

Immunization and challenge of guinea pigs. Groups of Hartley guinea pigs were immunized intramuscularly (i.m.) with 0.1-ml to 0.5-ml doses of either spores or log-phase vegetative cells, or with 0.5-ml doses of the human anthrax vaccine, MDPH-PA (19), prepared by the Michigan Department of Public Health. Eight weeks after the first immunizations, guinea pigs were challenged i.m. with spores of the virulent B. anthracis Ames strain (100 spores = 1 LD_{50}). The numbers of animals that died within 2 weeks after challenge were recorded.

Lethality of B anthracis FD111, FD112 and UM23-1 for A/J mice. A/J mice were injected subcutaneously (s.c.) in groups of 20 with 10^4, 10^5, 10^6, 10^7, or 10^8 CFU of B. anthracis FD111, FD112, or the Tc^s, Aro^+ parent strain, UM23-1. Mortalities were noted for 14 days after injection.

Immunization and challenge of mice. Groups of CBA/J or A/J mice were immunized s.c. with 0.2-ml doses of either spores, log-phase vegetative cells, or MDPH-PA. Eight weeks after the first immunizations, mice were challenged s.c. with 0.2-ml of B. anthracis Ames strain spores. The LD_{50} of Ames spores are <1 spore (calculated LD_{50} = 0.5 spore) for A/J mice and 35 spores for CBA/J mice.

Deaths within 2 weeks after challenge were noted.

Serological studies. Prechallenge sera from guinea pigs (15) and mice (25) were examined for antibody to PA by using an enzyme-linked immunosorbent assay (ELISA) (15). Anti-PA titers were determined (8).

RESULTS AND DISCUSSION

PA-producing transformants of B. subtilis DB104. Approximately 3000 Km^r colonies (600 per μg of DNA) were isolated after transformation of B. subtilis DB104 with pPA101 DNA. Over 400 colonies were then transferred to immunoassay agar (17) to identify putative PA-producing clones. All of the tested clones were surrounded by immunoprecipitin halos. Eighteen of the clones were randomly selected and serially subcultured five times. They remained halo-positive. Plasmid DNA from these 18 clones was isolated (3) and analyzed by agarose gel electrophoresis (9,16). The plasmid DNAs from all 18 strains appeared identical in size to the 7.8-kb pPA101 plasmid (data not shown).

Plasmids from two of the 18 clones described above, PA7 and PA8, were compared with the pPA101 plasmid in agarose gel electrophoresis. Both PA7 and PA8 were identical in electrophoretic profile to pPA101, either undigested or after digestion with BamHI, HindIII, EcoRI, or PvuII (data not shown). Thus, it was apparent that B. subtilis PA7 and PA8 possessed plasmid pPA101. B. subtilis PA7 was examined further; it produced PA (9) that was biologically (6,21) and serologically (9,24) identical to the PA produced by B. anthracis Sterne and by B. subtilis PA1.

Immunization of guinea pigs with B. subtilis PA-producing recombinant strains. The protective efficacy of the asporogenic, PA-producing B. subtilis recombinant strains, PA1 and PA2 (9), was tested. Two immunizations at 0 and 4 weeks with 10^8 or 10^9

CFU of the nonrecombinant, pUB110-containing strain, B. subtilis BST1, gave no protection from an i.m. challenge of 20 LD_{50} of B. anthracis Ames spores at 8 weeks. In contrast, with PA1 and PA2, guinea pigs were completely protected from lethality by two doses of 10^8 or 10^9 CFU. B. anthracis Sterne cells completely protected the guinea pigs with two doses of 10^6 or 10^7 CFU; however, one animal injected with 10^8 CFU died.

The safety and efficacy of B. subtilis PA1 and PA2 vegetative cells, B. subtilis PA7 spores, B. anthracis Sterne spores, and MDPH-PA were then compared in guinea pigs (Table 2). Two doses of 10^7 CFU of Sterne spores completely protected against the Ames spore challenge. However, 11 of 33 guinea pigs were killed by the immunization. In contrast, two doses of 10^9 CFU of PA1 or PA2 vegetative cells protected 95% of the animals and caused no deaths. Two injections of 10^{10} CFU of PA7 spores killed two guinea pigs, but completely protected the remaining 18 challenged animals. Although immunization with MDPH-PA elicited the highest anti-PA titers, only 75% of the immunized animals were protected from challenge (Table 2). These data confirm previous reports (15,23,25) that there is no strict correlation between anti-PA titers and protection against spore challenge. The data also demonstrate that the PA-producing B. subtilis recombinant strains protect guinea pigs from virulent spore challenge and that they are safer than B. anthracis Sterne spores.

Immunization of mice with B. subtilis PA-producing recombinant strains. Like guinea pigs, CBA/J mice immunized with MDPH-PA

develop high titers to PA (25). Unlike guinea pigs, however, these vaccinated mice are not protected against virulent anthrax spore challenge (25). Furthermore, doses of B. anthracis Sterne spores required to protect CBA/J mice from virulent challenge are only slightly less than those which kill some of the mice (25). Since mice differ from guinea pigs in their response to anthrax vaccines, we tested the PA-producing recombinant strains for safety and efficacy in CBA/J mice. Virtually no protection against Ames spore challenge was seen in mice immunized with three doses of MDPH-PA; B. subtilis strains DB104 or BST1, which do not possess the PA gene; or B. anthracis ΔSterne-1, which does not contain the genes for PA, LF or EF (Table 3). B. subtilis strains PA1, PA2, and PA7 were as efficacious as B. anthracis Sterne vegetative cells, protecting ≤70% of the mice against Ames challenge. Thirty-four per cent of the mice vaccinated with Sterne vegetative cells were killed by the immunization, whereas no vaccine-related mortalitites occurred in the other immunization groups (Table 3). Both the Sterne spore vaccine and strain PA2 protected 100% of CBA/J mice against challenge with B. anthracis strain Vollum 1B (25). Thus, the PA-producing B. subtilis recombinant strains were safe and effective prototype live anthrax vaccines in CBA/J mice, as they were in Hartley guinea pigs.

Characterization and reversion of B. anthracis Aro⁻ strains. Two mutants derived by Tn916 mutagenesis and deficient in synthesis of aromatic compounds were described previously (11). The B. anthracis Aro⁻ strains, FD111 and FD112, grew substantially

slower than the parent UM23-1 strain, even in the nutritionally rich AGM. Doubling times were 24 min, 80 min, and 64 min, respectively, for UM23-1, FD111, and FD112.

It is essential for live, attenuated vaccines to be stable and not revert to the more virulent, parental phenotype. Therefore, we tested B. anthracis FD111 and FD112 for reversion from Aro$^-$ to Aro$^+$ and Tc^r to Tc^s. No reversion was detected when cultures were grown in the presence of tetracycline. In medium lacking tetracycline, no revertants were detected during logarithmic growth. However Aro$^+$ and Tc^s revertants appeared after the cultures entered stationary phase (Table 4). The reason for reversion only after cessation of logarithmic growth was not investigated. Neither the Aro$^-$ nor the Tc^r phenotypes reverted separately, suggesting that excision of Tn916 regenerated a functional gene in the B. anthracis aromatic biosynthetic pathway and resulted in loss of Tn916 from the cell. There was no evidence of either transposition of Tn916 to another site on the genome, or loss of Tn916 without regeneration of functional DNA. All revertants were phenotypically indistinguishable from the parent UM23-1 on several minimal and complex media, with or without tetracycline. The in vivo rates of reversion of B. anthracis FD111 and FD112 after injection into mice or guinea pigs were not examined in these studies.

Immunization of guinea pigs with B. anthracis Aro$^-$ strains. Immunization with either one dose of 10^9 or two doses of 10^8 CFU of B. anthracis FD111 or FD112 cells gave strong protection to

guinea pigs against an Ames spore challenge without killing any animals during vaccination (Table 5). Immunization with the same doses of B. anthracis UM23-1 also protected guinea pigs from challenge, but 10^9 CFU killed 8 of 17 animals.

Lethality of B. anthracis FD111, FD112, and UM23-1 for A/J mice. A/J mice are quite susceptible to lethal infection by various strains of B. anthracis, including the toxinogenic, nonencapsulated Sterne vaccine strain (26). Injection of as few as 10^4 CFU of B. anthracis UM23-1 vegetative cells killed 100% of A/J mice, whereas injections of up to 10^8 CFU of B. anthracis FD111 or FD112 cells killed none of the mice. Thus the Aro$^-$ strains were substantially less virulent in mice than the parent UM23-1 strain.

Immunization of mice with B. anthracis Aro$^-$ strains. A/J mice are unable to be immunized with B. anthracis Sterne, since Sterne spores are lethal for A/J mice at immunogenic doses (26). In contrast, the A/J mice were partially protected from a challenge of Ames spores by immunization with $\geq 10^7$ CFU of B. anthracis FD112 cells (Table 6). Although avirulent for the mice, the Aro$^-$ strains were probably able to replicate sufficiently to generate a protective immune response against a subsequent, virulent B. anthracis spore challenge.

Finally, both A/J and CBA/J mice were immunized with 10^7 or 10^8 B. anthracis FD111 cells. Two doses of FD111 cells protected $\geq$80% to CBA/J mice against challenge with 6 LD_{50} of Ames spores (Table 7). One or two doses of FD111 cells protected $\leq$70% of A/J

mice against challenge with approximately 60 LD_{50} of Ames spores (Table 7). The Aro$^-$ strains were the first vaccines tested which were able to protect A/J mice against challenge with a fully virulent strain of B. anthracis.

Hoiseth and Stocker (7) used Tn10 mutagenesis to obtain Aro$^-$ strains of Salmonella typhimurium as live vaccines. Similarly the objectives of our initial research with B. anthracis and Tn916 (11) were to demonstrate the utility of transposon mutagenesis in B. anthracis and to generate Aro$^-$ mutants as prototype live vaccines. The immunization studies described here in both the mice and guinea pigs indicate that B. anthracis FD111 and FD112 were efficacious as live vaccines and were far safer and less virulent than the B. anthracis UM23-1 parent strain, which is a Str^r, Ura$^-$ variant of the veterinary vaccine Sterne (Weybridge) strain. Obvious drawbacks of these prototype, Aro$^-$ live vaccines include the possession of a self-transmitting tetracycline resistance factor and their ability to revert to the Aro$^+$, Tc^s parental phenotype. These drawbacks would be eliminated in B. anthracis Aro$^-$ mutants developed by chemical mutagenesis or by mutagenesis with a non self-transmitting transposon that does not excise precisely to restore the function of the insertionally inactivated segment of DNA

These studies demonstrate that new, live vaccines can be developed against anthrax which are efficacious against challenge from the highly virulent B. anthracis Ames strain, and which are safer in experimental animals than the live B. anthracis Sterne

veterinary vaccine strain. Future studies on immunization as well as the elucidation of the mechanisms of immunity to anthrax will facilitate our goal of a safer, more efficacious, and long-lasting human vaccine against anthrax.

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TABLE 1. Bacterial strains used

Strain	Plasmids	Relevant Characteristics[a]	Source or derivation
B. anthracis			
Ames	pXO1, pXO2	PA$^+$, LF$^+$, EF$^+$, Cap$^+$	USDA[b]
Sterne	pXO1	PA$^+$, LF$^+$, EF$^+$	USAMRIID[c]
ΔSterne-1[d]	None		USAMRIID; 17
UM23-1[e]	pXO1	PA$^+$, LF$^+$, EF$^+$ Str^r, Ura$^-$	C. B. Thorne
FD111[f]	pXO1	PA$^+$, LF$^+$, EF$^+$, Tc^r (Tn*916*), Aro$^-$, Str^r, Ura$^-$	USAMRIID; 11
FD112[f]	pXO1	PA$^+$, LF$^+$, EF$^+$, Tc^r (Tn*916*), Aro$^-$, Str^r, Ura$^-$	USAMRIID; 11
B. subtilis			
BST1[g]	pUB110	Km^r, Asporogenic	USAMRIID; 9
PA1	pPA101[h]	Km^r, Asporogenic, PA$^+$	USAMRIID; 9
PA2	pPA102[h]	Km^r, Asporogenic, PA$^+$	USAMRIID; 9
DB104	None	Sporogenic	Roy Doi; 12
PA7[i]	pPA101	Km^r, Sporogenic, PA$^+$	This study
PA8[i]	pPA101	Km^r, Sporogenic, PA$^+$	This study

[a] Abbreviations: Aro, aromatic biosynthetic pathway; PA, protective antigen; LF, lethal factor; EF, edema factor; Cap, capsule; Km, kanamycin; Sm, streptomycin; Tc, tetracycline; Ura, uracil.

[b] U.S. Department of Agriculture, Ames, Iowa.

[c] U.S. Army Medical Research Institute of Infectious Diseases.

[d] Derived by serial subculture of *B. anthracis* Sterne at 42.5°C to cure the cells of pXO1. ΔSterne-1 is nonencapsulated and nontoxinogenic.Se Derived from the veterinary vaccine Sterne (Weybridge) strain.

[f] Derived by conjugation of B. anthracis strain VNR-1-1 (containing Tn916) with B. anthracis strain UM23-1 and selection for Aro$^-$, Tc^r transposition mutants.

[g] B. subtilis BGSC 1S53 transformed with pUB110, a 4.5-kb plasmid vector encoding kanamycin resistance.

[h] Recombinant plasmid containing the B. anthracis PA gene ligated into pUB110; transformed into competent B. subtilis BGSC 1S53.

[i] B. subtilis DB104 transformed with pPA101 according to methods described.

TABLE 2. Immunization of guinea pigs with B. subtilis PA1, PA2, and PA7

Immunization[a]	Doses	No. survived / no. challenged	(%)[b]	Serologic response[c]
PBS	3	0/16	(0)	<10
MDPH-PA	3	12/16	(75)	20,535
B. anthracis Sterne				
10^6 CFU	1	11/15	(73)	392
10^6 CFU	2	14/16	(88)	10,700
10^7 CFU	1	10/12[d]	(83)	4,641
10^7 CFU	2	10/10[e]	(100)	3,981
B. subtilis PA1				
10^8 CFU	2	10/14	(71)	13,895
10^9 CFU	1	0/16	(0)	1,225
10^9 CFU	2	19/20	(95)	5,623
B. subtilis PA2				
10^8 CFU	2	5/14	(36)	1,912
10^9 CFU	1	1/16	(6)	277
10^9 CFU	2	18/19	(95)	3,450
B. subtilis PA7				
10^8 CFU	2	1/17	(6)	667
10^9 CFU	1	0/17	(0)	115
10^{10} CFU	2	18/18[f]	(100)	9,380

[a]Guinea pigs were immunized i.m. at 0 weeks (1 dose), 0 and 4 weeks (two doses), or 0, 2 and 4 weeks (three doses). Guinea pigs were immunized with vegetative preparations of PA1 or PA2, and with spore preparations of Sterne or PA7.

[b] Animals were challenged 4 weeks after the last vaccine dose with 43 LD_{50} of Ames spores.

[c] Reciprocal geometric mean anti-PA ELISA titers of prechallenge sera.

[d] Five guinea pigs died from the immunization.

[e] Six guinea pigs died from the immunization.

[f] Two guinea pigs died from the immunization.

TABLE 3. Immunization of CBA/J mice with B. subtilis PA2 and PA7

Immunization[a]	Spore Challenge Dose (LD_{50})[b]	No. survived / No. challenged	(%)	Serological response[c]
PBS	6	0/5	(0)	<10
PBS	17	0/5	(0)	<10
MDPH-PA	10	1/10	(10)	100,000
MDPH-PA	20	0/20	(0)	67,608
B. anthracis				
ΔSterne-1	6	0/10	(0)	<10
Sterne[d]	6	8/12	(67)	3,162
Sterne[d]	17	6/9	(67)	12,589
Sterne spores	17	9/20	(45)	2,818
B. subtilis				
DB104	17	0/10	(0)	<10
BST1	6	0/10	(0)	<10
PA2	6	8/12	(67)	4,217
PA2	17	2/3	(67)	10,000
PA7	17	7/10	(70)	5,623

[a] Mice were immunized s.c. three times at 2-week intervals. Mice were immunized with vegetative preparations of PA2, BST1, ΔSterne-1, or Sterne; and with spore preparations of DB104, PA7, and Sterne.

[b] LD_{50} for CBA/J mice is 35 B. anthracis Ames spores.

[c] Reciprocal geometric mean anti-PA ELISA titers of prechallenge sera.

[d] Log phase vegetative cells. Eleven of 32 mice died after the first immunization.

Table 4. Reversion of B. anthracis FD111 and FD11 in the absence of tetracycline[a]

Strain	Culture age	%Tc[s]	% Aro[+]
FD111			
	0 time	<0.01%[b]	<0.01%
	30 h[c]	<0.01%	<0.01%
	48 h[d]	0.49%	0.49%
FD112			
	0 time	<0.01%	<0.01%
	24 h[e]	<0.01%	<0.01%
	42 h[d]	1.06%	1.06%

[a] No reversion to the Tc[s] or Aro[+] phenotypes was detected when cells were grown with tetracycline.

[b] No revertants were detected among 10^4 colonies examined.

[c] Sample taken during log phase, after 19.1 generations

[d] Sample taken during stationary/death phase of culture.

[e] Sample taken during log phase, after 18.3 generations.

TABLE 5. Immunization of guinea pigs with *B. anthracis* Aro⁻ strains

Immunization[a]	Doses	No. survived / no. challenged	(%)[b]	Serological response[c]
PBS	2	2/15	(13)	<10
B. anthracis FD111				
10^8 CFU	2	17/17	(100)	9,345
10^9 CFU	1	16/16	(100)	1,540
B. anthracis FD112				
10^8 CFU	2	16/16	(100)	6,978
10^9 CFU	1	13/15	(87)	1,848
B. anthracis UM23-1				
10^8 CFU	2	17/17	(100)	6,225
10^9 CFU[d]	1	9/9	(100)	5,275

[a] Guinea pigs were immunized i.m. at 0 and 4 weeks with 10^8 CFU, or at 4 weeks with 10^9 CFU of vegetative cells.

[b] Animals were challenged 4 weeks after the last vaccine dose with 16 LD_{50} of Ames spores.

[c] Reciprocal geometric mean anti-PA ELISA titers of prechallenge sera.

[d] Eight guinea pigs died from the immunization.

TABLE 6. Immunization of A/J mice with *B. anthracis* FD112

Immunizing Dose (CFU)[a]	No. survived no. challenged	(%)[b]
10^4	0/20	(0)
10^5	3/20	(15)
10^6	3/19	(16)
10^7	9/20	(45)
10^8	8/20	(40)

[a] Mice were immunized s.c. at 0 and 4 weeks.

[b] Mice were challenged at 8 weeks with approximately 100 LD_{50} of Ames spores.

TABLE 7. Immunization of A/J and CBA/J mice with B. anthracis FD111

Mouse Strain	FD111 Immunization[a] CFU	No. Doses	Ames spore challenge[b] Dose (LD_{50})	Survival	(%)
A/J					
	(PBS)	1	60	0/10	(0)
	10^7	1	60	7/10	(70)
	10^7	2	60	5/9	(56)
	10^8	2	60	4/6	(67)
CBA/J					
	(PBS)	1	6	1/20	(5)
	10^7	1	6	6/10	(60)
	10^7	2	6	10/10	(100)
	10^8	1	6	4/10	(40)
	10^8	2	6	8/10	(80)

[a] Mice were immunized s.c. at 0 and 4 weeks, or at 4 weeks alone.

[b] Mice were challenged at 8 weeks.